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Optimizing glutaraldehyde crosslinking of collagen: effects of time, temperature and concentration as measured by shrinkage temperature

J. M. RUIJGROK, J. R. DE WIJN
Department of Biomaterials, bld 55, Leiden University, Rijnsburgerweg 10, 2333 AA, Leiden, The Netherlands

M. E. BOON
Leiden Cytology and Pathology Laboratory, Groenesteeg 61–65, 2312 SP, Leiden, The Netherlands

Glutaraldehyde (GA) crosslinking (fixation) of collageneous tissues is a widely used method for the preparation of implantible tissues to be used as biomaterials. In an attempt to optimize the fixation process, experiments were carried out with two types of collagen (native collagen membrane and synthetic collagen sheet) to study the effect on crosslinking of temperature, GA concentration and fixation time. Secondly, stimulation of GA diffusion was studied and finally, a procedure of low T-presoaking followed by brief exposure to high temperatures was investigated. As a measure of the degree of crosslinking the shrinkage temperature (T_s) was determined. Temperature (20 °C or 45 °C), concentration (0.1% or 1.0%) or fixation time (4 or 24 h) were found to be positively correlated with the T_s of the collagen sheets. Whereas untanned collagen exhibits a T_s of around 60 °C, short-term (1 or 5 min), high-temperature (50 °C) fixation with a 0.1% GA solution caused the shrinkage temperature to increase to 72 °C and 85.1 °C, respectively. Fixation with 0.01% GA for 5 min at 50 °C appeared equally effective as 1 min with 0.1% GA ($T_s = 70$ °C). Microwave irradiation showed to be slightly more effective in enhancing the crosslinking process compared with conventional heating. Surprisingly, at any combination of temperature, concentration and fixation periods of 4 h or 24 h, an increased T_s towards the central regions of the collagen was observed. Soaking the samples at 20 °C (1 h) or at 0 °C (3 h) with subsequent short-time heating to 45 °C caused an almost equal rise in T_s throughout the collagen samples and is therefore recommended for preparing implantable tissues.

1. Introduction

Glutaraldehyde (GA) crosslinking of collageneous tissues for implant purposes significantly reduces antigenicity and biodegradation [1]. Other methods of crosslinking are less efficient than glutaraldehyde in generating chemically, biologically and thermally stable crosslinks. However, there have been reports of the failure of GA tanned bioprostheses (e.g. the Dardik umbilical vein [2, 3].

Aqueous solution of GA are mixtures of free aldehyde, mono- and dihydrated monomeric glutaraldehyde, monomeric and polymeric cyclic hemiacetals and various alpha, beta, unsaturated polymers which are in equilibrium. The content of free aldehyde is usually not more than 4% [4]. Because of the complex nature and reactivity of GA solutions relatively little is known about the reaction products formed during crosslinking and the optimum crosslinking conditions [5, 6]. Although GA reacts quickly and irreversibly, mainly with amino groups, it penetrates slowly into tissues [7], therefore the accessibility to the core regions of densely packed collagen fibres often presents a problem. Especially in bulky tissues such as skin and vascular prostheses, an inadequate penetration of GA may lead to an uneven distribution of crosslinks throughout the tissue. It is thought that this may lead to enhanced biodegradation, antigenicity and loss of mechanical function [1]. The problem of inadequate penetration of fixatives is also encountered while processing tissues for histology and pathology. A recent technique employed in this field is the use of microwave irradiation to enhance the speed of fixation of the specimens [8]. During different steps of histoprocessing, the tissue or cells are irradiated, typically for less than 2 min, in which time the tissue reaches a temperature of 45 to 60 °C. At this temperature proteins are known to change their tertiary structure, thereby exposing reaction sites unexposed at room

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temperature [9]. In addition, GA monomer formation from polymeric forms will be enhanced by heating. Although heating GA solutions for a longer period (days to months) or at temperatures higher than 90 °C, will enhance the formation of these unwanted polymers, short-time (less than 1 h) heating below 90 °C will promote the formation of the GA monomer from the various polymeric forms, rather than the production of polymers [10].

The concentration of free GA monomer will rise from 4% at room temperature to 35% at 50 °C [4]. Furthermore, at 50 °C diffusion rates will be higher than at room temperature, and the reaction between the fixative and the amino groups will be accelerated [11], permitting shorter fixation times with lower concentrations of the fixative [8]. Finally, the small GA monomers (molecular weight 100) are expected to diffuse more rapidly than large GA polymers.

Thus GA processing of bioprostheses at higher temperatures could have at least two advantages. GA is rather toxic even in relatively low concentration, and GA processed bioprostheses are known to be prone to calcification in the long term, which has been related to the amount of GA introduced into the collagen [12]. Therefore, a brief exposure to a low GA concentration could lead to fewer toxic products thereby reducing the tendency to calcify.

Second, the polymeric forms of GA may, once introduced into the implant, degrade, long after implantation, into oligomers and monomers, which after diffusion into the surrounding tissue, may give rise to inflammatory reactions, thereby shortening the lifespan of the implant. Furthermore, GA polymers are thought to be responsible for the slow penetration of GA into the collagen matrix, either by steric hindrance, or by the formation of nucleation sites to which further GA molecules may attach [1]. Therefore one approach to optimize GA crosslinking of dense collageneous tissues could be the application of heat. Recent experiments employing sheep dermal collagen, showed that short time GA fixation at temperatures up to 50 or 60 °C, can result in an increase in shrinkage temperature of more than 20 °C within 5 min [13].

The density of the crosslinks formed can be studied by determination of the rise in shrinkage temperature (the temperature at which the collagen shrinks during gradual heating), a method originally used in the leather industry [12]. Earlier studies on enzymatic digestion and resorption *in vivo* [1, 14], showed that the higher the shrinkage temperature of the material, the less susceptible the collagen matrix is to enzymatic attack by enzymes such as collagenase, and the longer the lifespan of the implant *in vivo*.

In this paper, five experiments are described, carried out to find the relevant parameters for optimizing the crosslinking process. The first experiment (A, see Table I) was conducted in an attempt to obtain a high shrinkage temperature using a minimal amount of glutaraldehyde, and, as a result of the use of low GA-concentration, a short fixation time and high temperature. We also compared the effects of conventional (external) heating employing a waterbath with internal heating using a microwave oven.

The second experiment (B) was performed to study the diffusion of GA into a collagen matrix under the typical conditions used in the preparation of collageneous implants (room temperature, high concentration and long exposure times). We developed a multilayer model consisting of ten synthetically formed collagen sheets. In our model, the influence of temperature, concentration and fixation time on the distribution of the T_s can be determined from the outer layers to the core regions of the collagen (Fig. 1).

Because GA is known to penetrate slowly but to react quickly with amino groups, we assumed that an inadequate diffusion of GA may lead to an uneven distribution of crosslinks (and thus T_s) throughout the thickness of a collagen sheet.

An additional test (C) was performed to check whether the findings of experiment (B) with the multilayer model could also be detected in native collagen membrane.

In the fourth experiment (D) the effect of short-time high-temperature crosslinking using a low GA concentration was used to study the degree of crosslinking and the distribution of crosslinks throughout the multilayer model.

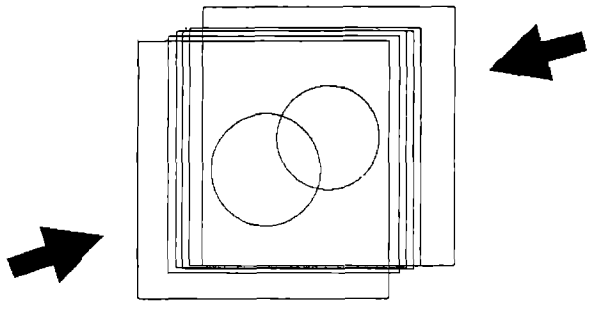

Figure 1 The multilayer model.

TABLE I Description of the five experiments

Experiment	Aim	Material
A	Adequate crosslinking using minimal amounts of GA, high temperature, and short fixation times	Native collagen membrane
B	To reveal factors of influence on diffusion of GA into a multilayer collagen matrix	Synthetic collagen fleece
C	To study diffusion of GA into native collagen using a 4 layer model	Native collagen membrane
D	To study the distribution of crosslinks throughout collagen matrix using conditions as in exp. A	Synthetic collagen fleece
E	To study effect of heating on collagen crosslinking after prior GA soaking	Synthetic collagen fleece

In the last experiment (E), we investigated the importance of the diffusion of GA in the total fixation process by soaking the multilayer model in a GA solution at low temperature, allowing for penetration with minimum crosslinking. The presoaking step, was followed by brief heating, either by microwave irradiation or by conventional heating in a waterbath.

2. Materials and methods

2.1. Collagen

Native processed porcine collagen membrane (Bioplex Medical Corporation, Vaals, The Netherlands) was used as-received in experiments A and C. Synthetic collagen sheets made of collagen dispersion (Naturin Gmbh, Weinberg, Germany) were used in experiments B, D and E.

2.2. Fixation solutions

Glutaraldehyde (25% w/v, Sigma Chemicals) was purified by vacuum distillation and stored as an 8% w/v solution at $-18\,^{\circ}C$ until use. Ultraviolet absorption spectra were taken to ensure the polymer to monomer ratio was as low as possible in the starting solution (< 0.2) by determining the ratio of absorption at 235 nm and 280 nm, respectively.

2.3. Heating methods

2.3.1. Microwave heating

A Biorad H 2500 microprocessor-controlled microwave oven (600 W) was used. The power was set at 60% (360 W) and a waterload of 500 ml demineralized water was placed in a corner to absorb excess radiation. The oven uses a thermocouple for temperature measurement and control. To ensure adequate temperature measurement the temperature was checked using a fibre optic thermometer (Asea, Rotterdam), the only device which can be reliably used inside a microwave oven. The difference between the temperature reading of the thermocouple and that of the fibre optic thermometer was corrected by recalibrating the microprocessor.

The glutaraldehyde (GA) solution was stirred by a battery-powered back and forth moving device made out of a toy construction kit (Fisher Technik (FT), 7241 Tumlingen, Germany), as previously described [15] to ensure a homogeneous temperature throughout the heated solution.

2.3.2. Waterbath heating

The waterbath (Tamson, Zoetermeer) was set to minimize temperature fluctuation. The GA solution was stirred in the same way as in the microwave experiments.

2.4. Determination of shrinkage temperature (T_s)

In experiment A the T_s was determined using a dimensional change test apparatus, commonly exploited in the leather industry [16]. In this device strips of collagen measuring 50×3 mm are mounted in a waterbath between a fixed point and a wire running over a pulley. A preload of 0.04 N is applied by a small copper weight connected to the free end of the wire. The pulley drives a pointer over a dimensional scale. The water is gradually warmed up from 20 to $90\,^{\circ}C$ ($2\,^{\circ}C$/min). Any change in length of the collagen is transformed into a deflection of the pointer. The temperature at which maximum deflection occurs is recorded as the shrinkage temperature.

In experiment B, C, D, and E, the shrinkage temperature was determined using differential scanning calorimetry (DSC, Dupont Instruments 910, heating rate set at $2\,^{\circ}C$/min) on each separate sheet of the multilayer model. The observed transition was treated as a glass transition and the mid-temperature recorded as the shrinkage temperature. As established in earlier studies, both methods for determining the T_s give comparable results [17].

2.5. Experiment A

In the first experiment (A) native collagen membrane was crosslinked for 1 or 5 min at a temperature of $50\,^{\circ}C$ and with a 0.1% concentration of GA, employing microwave irradiation and conventional heating using a waterbath. The high tanning temperature was chosen safely below the natural shrinkage temperature of untanned native collagen (around $62\,^{\circ}C$). Three pieces of collagen (50×3 mm) were fixed simultaneously in 300 ml of a 0.01% or a 0.1% GA solution in phosphate buffered saline (PBS) at pH 7.4, for 1 min or 5 min, using a microwave oven or a waterbath. A total of 15 pieces per method were obtained. Under favourable conditions 26 moles of GA (2603 g) are involved in unipoint or multipoint bonding with 26 moles (100 kg) of reactive amino groups of collagen [18]. Three collagen strips of 0.126 g, could, therefore, theoretically react with 9.84 mg GA. The 0.01% GA solution contained 30 mg of GA, a more then threefold excess.

2.6. Experiment B

A multilayer model for use in experiment B, D, and E consists of 10 layers of thin (0.04 mm) collagen sheet, made of a homogeneous collagen dispersion. The sheets were enclosed between two plexiglass covers in which a circular opening was made (Fig. 1). After immersion in PBS and deaeration under vacuum, the sides of the cell were sealed with UV-curable adhesive to prevent the solutions intruding between the sheets from a lateral direction. The size of the sheets (12 cm^2, 24 mg) was chosen so as to allow measurement of the shrinkage temperature using DSC. The multilayer samples were immersed in 80 ml GA solution of 1.0% or 0.1% (w/v) in PBS (pH 7.4). The shrinkage temperature of the untanned collagen used in this experiment is around $50\,^{\circ}C$, therefore the temperature was set at $20\,^{\circ}C$ or $45\,^{\circ}C$. The fixation time was set at 4 h or 24 h.

In a separate test, an old glutaraldehyde solution was used, consisting mainly of GA polymers (polymer to monomer ratio > 2.0). In this test 1.0% GA was used, for 24 h at 45 °C.

Each collagen sandwich contained 240 mg collagen which could, when ideally crosslinked, react with about 6 mg of GA [18]. The 0.1% GA solutions contained 80 mg of GA, a more then 10-fold excess. After tanning, the cell was dismantled, the sheets separated, extensively rinsed with excess demineralized water to stop further crosslinking, and stored at 4 °C until determination of the shrinkage temperature by DSC.

2.7. Experiment C

Four native collagen membranes were stacked in a cell similar to experiment B and crosslinked for 24 h at 45 °C (1.0% GA in PBS, pH 7.4).

After tanning the membranes were extensively washed in tapwater to stop further crosslinking. After embedding in a polyethylenglycol/alcohol medium (Kryofix), the membranes were cut into slices of 5 μm parallel to the surface, employing a freezing microtome technique. From every second slice the shrinkage temperature was determined using DSC (as in experiment B).

2.8. Experiment D

The effect of short-time high-temperature fixation on T_s was studied in the multilayer model. The model was tanned for 5 min at 45 °C in 300 ml GA solution (1.0% in PBS, pH 7.4), employing both methods of heating. After tanning, T_s was determined as in experiment B.

2.9. Experiment E

The collagen sandwiches were soaked for 1 h at room temperature or 3 h at 0 °C in the GA solution (300 ml, 0.1% in PBS, pH 7.4). Then, the model was exposed to either microwave or waterbath heating. The GA solution was irradiated continuously inside the microwave oven at 60% of the maximum power (360 W), reaching the final temperature of 45 °C within 1 min 30 s (room temperature) or 3 min (0 °C). In the waterbath the presoaked model was placed in a GA solution preheated to 45 °C. After tanning, the model was treated and T_s determined as in experiment B.

3. Results

3.1. Experiment A

Fig. 2 shows the effect of short-time (1 and 5 min) high-temperature (50 °C) fixation on the shrinkage temperature of the collagen membrane, derived from bovine peritoneum.

The shrinkage temperature of untanned native collagen membrane (62.3 °C), and the maximum attainable shrinkage temperature employing glutaraldehyde tanning is also shown (≈ 91 °C). The latter temperature was established using high-temperature (50 °C) fixation for 9 days at 0.1% GA.

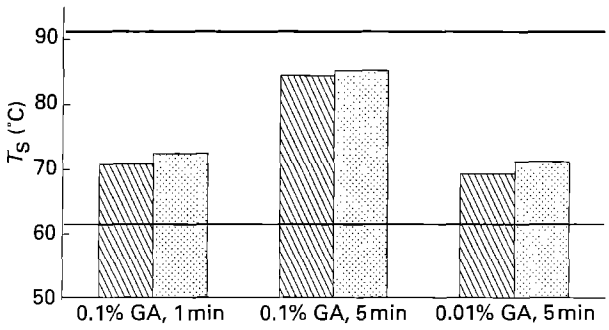

Figure 2 Short-time high-temperature fixation of native collagen membrane, comparing microwave irradiation (▨) with conventional heating (▩). The horizontal lines represent the shrinkage temperature of untanned native membrane (62.3 °C) and the maximum attainable shrinkage temperature (92 °C)

Fixation using 0.1% GA at 50 °C for 1 min caused a rise in shrinkage temperature of 8–10 °C, whereas after 5 min a rise of more than 22 °C was obtained.

A low concentration of 0.01% GA caused a rise in shrinkage temperature of around 8 °C within 5 min. Thus, crosslinking for 1 min using 0.1% GA was more or less as effective in stabilizing the collagen as 0.01% for 5 min. Remarkably, at 0.1% GA, a shrinkage temperature near maximum was obtained within 5 min of high temperature fixation.

Both methods of heating, microwave irradiation and waterbath, appeared to be effective in stimulating crosslinking, the microwave heating method showing only a slight but significant (Table II, $p < 0.05$) advantage over conventional heating in this experiment.

3.2. Experiment B

The results of the fixation experiments with synthetic collagen sheet are shown in Fig. 3.

In each graph, the shrinkage temperature is plotted against the position of the sheet within the cell. The positions are numbered from 1 to 10, resulting in "shrinkage temperature profiles". Each point of the profile consists of the averaged values of three samples crosslinked under the same conditions. Because the cells were open on both sides, sheet 1 can serve as a duplicate of sheet 10, sheet 2 as a duplicate of 9, etc. Sheet 5 and 6 are the central sheets. Notably, at each combination of temperature, concentration and fixation time an increasing T_s towards the central sheets was found, while T_s was lowest in the outer layers of the collagen (Fig. 3a–d). The difference in T_s between outer and inner sheet could be as high as 5 °C.

To compare the overall level of T_s, excluding the effect at the ends of the profiles, the values of the middle six positions were pooled (3 to 8) and the mean values compared using multivariate analysis of variance (MANOVA).

Compared with crosslinking at the mildest conditions of 20 °C and 0.1% concentration, an increase in any of the three parameters, (time, temperature, or concentration) is effective in raising the general level of shrinkage temperatures (Fig. 3a–d, Table III). A synergistic effect of the parameters appears to be

TABLE II Averaged shrinkage temperatures values (AVG) for experiment A. The *n*-values and the standard deviation (STD) are also shown (untan = untanned, max. = maximum tanning, mw = microwave heating, wb = waterbath heating)

	Tanning conditions							
	Untanned	0.01% mw	5 min wb	0.1% mw	1 min wb	0.1% mw	5 min wb	max
n =	6	13	13	12	12	14	14	7
AVG	62.3	71.1	69.3	72.4	70.9	85.1	84.4	91.1
STD	0.36	1.00	1.47	1.16	1.22	0.23	0.45	0.25

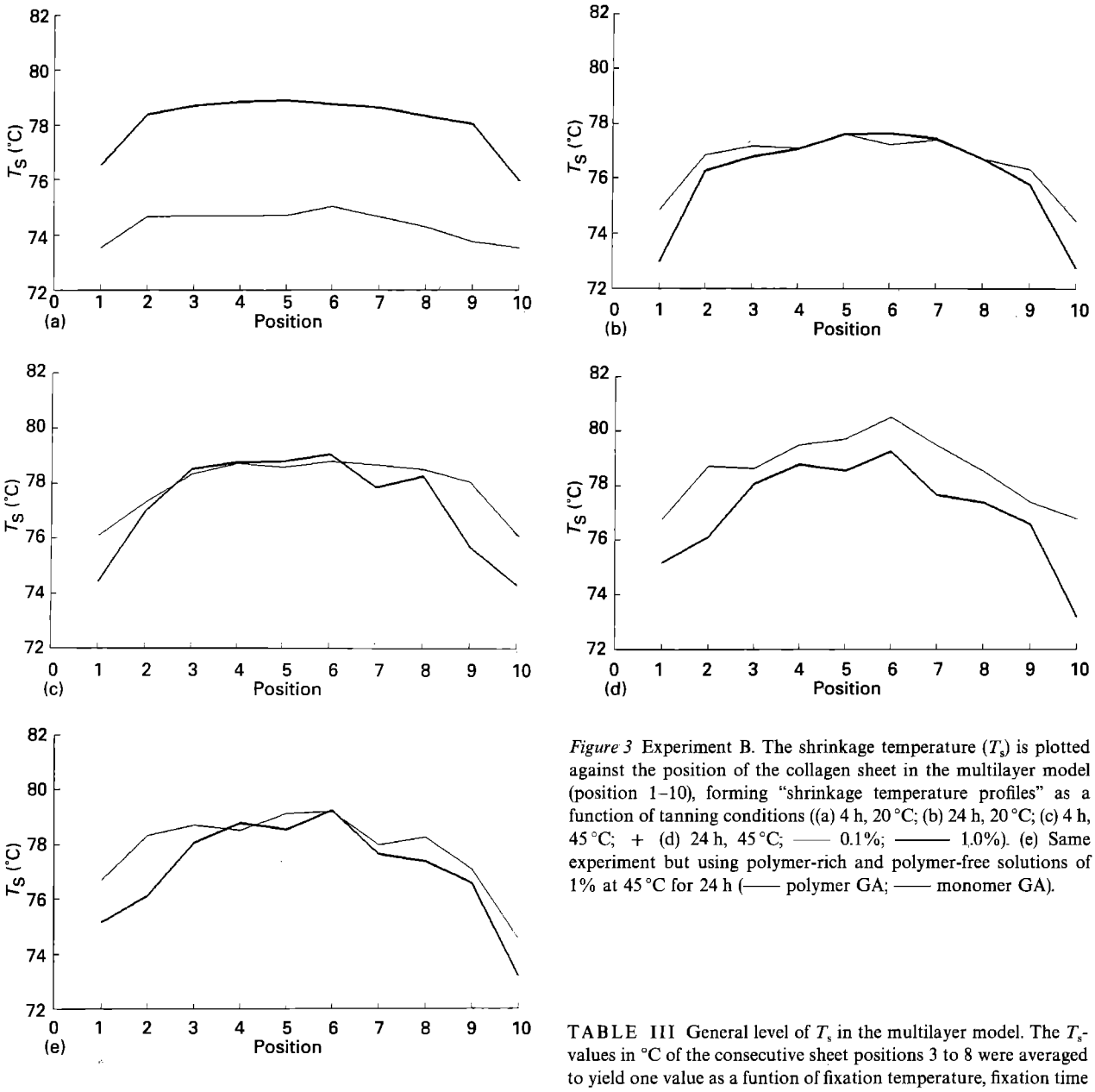

Figure 3 Experiment B. The shrinkage temperature (T_s) is plotted against the position of the collagen sheet in the multilayer model (position 1–10), forming "shrinkage temperature profiles" as a function of tanning conditions ((a) 4 h, 20 °C; (b) 24 h, 20 °C; (c) 4 h, 45 °C; + (d) 24 h, 45 °C; —— 0.1%; ——— 1.0%). (e) Same experiment but using polymer-rich and polymer-free solutions of 1% at 45 °C for 24 h (—— polymer GA; —— monomer GA).

present only in the 0.1% solutions, where prolonged fixation at higher temperature results in the maximum average shrinkage temperature of 80.07 °C. In 1% solution a maximum T_s of 78.88 °C is attained in 4 h at 20 °C.

The experiment performed at 45 °C for 24 h using 1.0% GA solution was repeated employing a GA solution containing mainly polymers (Fig. 3e). No significant difference could be observed in the general level of T_s for the two shrinkage temperature profiles.

In order to compare the shape of the profiles, the differences of the values of consecutive sheet positions

TABLE III General level of T_s in the multilayer model. The T_s-values in °C of the consecutive sheet positions 3 to 8 were averaged to yield one value as a funtion of fixation temperature, fixation time and GA concentration

	Fixation temperature and time			
	20 °C		45 °C	
Concentration	4 h	24 h	4 h	24 h
0.1%	74.67	77.18	78.63	79.45
1.0%	78.70	77.25	78.56	78.51

Significant increases:
0.1% → 1% GA, at 4 h, 20 °C ($p < 0.05$)
4 h → 24 h, at 0.1% GA, 20 °C ($p < 0.05$)
20 °C → 45 °C, at 0.1% GA, 4 h ($p < 0.05$)
Close to significant increase:
20 °C → 45 °C, at 0.1% GA, 24 h ($p = 0.06$)

were calculated and compared using MANOVA. Increasing temperature or concentration were found to produce more curvature of the profile (position 1–10). Trend tests showed that the deviation of linearity of the profile was significant ($p < 0.05\%$). The fixation period had no effect on the shape of the whole profile. When only the central part of the profile (position 3–8) was considered extending the tanning duration resulted in more curvature. Temperature and concentration did not significantly influence the shape of the central part of the T_s profiles.

3.3. Experiment C

In experiment C the crosslinking was studied in native collagen membrane of 400 μm thickness (Fig. 4). Here also, T_s increased, but less markedly, towards the inner regions of the outer membranes. Over a distance of 200 μm the shrinkage temperature was measured in the cut slices, increased from 81 to 83 °C. The experiment was performed three times. Because this model consisted of four membranes, the values of the outer halves of the outer membranes could be pooled. The inner halves of the outer membranes, as well as the inner two membranes, showed a consistent T_s of around 83 °C throughout the full thickness of the membrane matrix.

3.4. Experiment D

In experiment D the effects of short-time high-temperature heating was studied in the multilayer model, comparing both methods of heating (Figs 5 and 6).

In contrast with experiment B, in these short-term experiments the resulting T_s shows a strong tendency to decrease towards the inner regions of the sample. Here both methods of heating showed an almost equal inefficiency in enhancing diffusion of GA throughout the collagen sheet. Analysis of variance showed a tendency for the microwave oven to be slightly more efficient ($p = 0.06$) in this experiment.

In Fig. 6 it is shown that when the model was first presoaked in GA at low temperature with subsequent heating, the shrinkage temperature shows a quick and even rise in temperature, as shown by relatively flat curves.

In Figs 7 and 8 the results of experiment B and D are combined and supplemented with measurements of 20 min and 1 h in a waterbath. With increasing fixation times, the curve of the resultant T_s profile

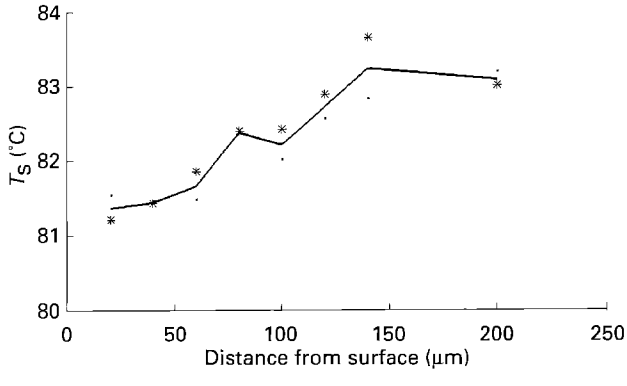

Figure 4 T_s of native collagen membrane as a function of distance to surface. The graph shows averaged values of 5 μm slices of the outer two membranes; the T_s value of the inner two membranes was constant (83 °C) throughout the full thickness.

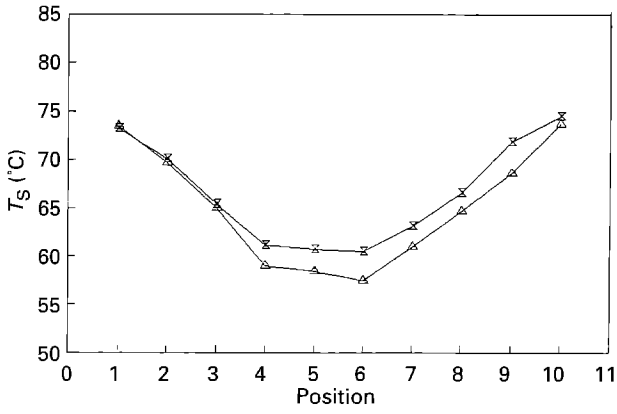

Figure 5 Comparison of the two methods of heating during short-time high-temperature fixation (0.1%, 45 °C, 5 min; △ waterbath, ⧖ microwave).

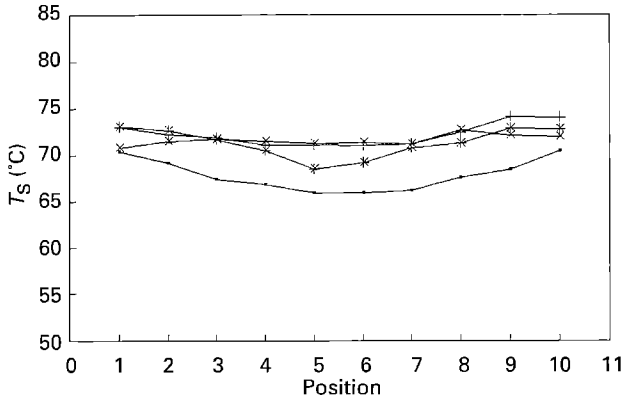

Figure 6 Effect of short-time high-temperature fixation on the T_s of GA presoaked collagen (-•-• 1 h, 20 °C; —✱— 1 min 30 s MW, 45 °C; + 1 min 30 s WB, 45 °C, —✕— 3 h 0 °C + 3 min 45 °C). The effect of only presoaking is also shown.

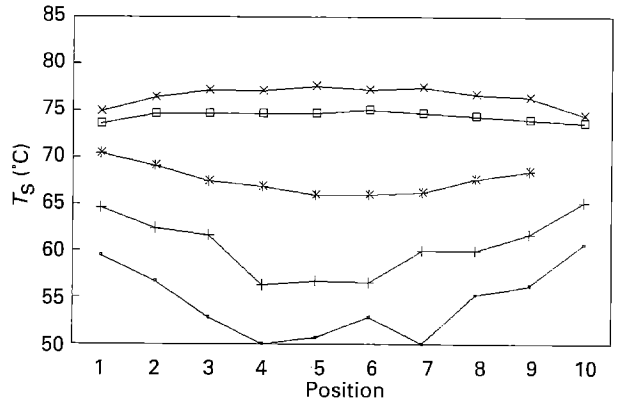

Figure 7 Shrinkage temperature profiles of multilayer models cross-linked during increasing time periods at 20 °C. The results of experiment B (4 and 24 h curves and D (5 min curves) are combined. In addition, 20 min profiles are shown (-•-• 5 min, —+— 20 min, —✱— 1 h, —□— 4 h, —×— 24 h).

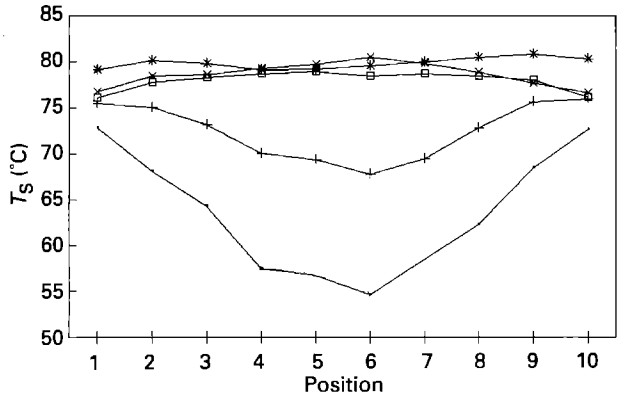

Figure 8 As Fig. 7, at a temperature of 45 °C.

changes from concave to convex. The profile transitions occur more rapidly at 45 °C than at 20 °C.

4. Discussion

In the field of histology and pathology limitation of exposure to GA is only of practical importance; during the preparation of implantable devices, however, the actual amount of GA and the nature and the distribution of the crosslinks formed are of vital importance: the less GA that is introduced into the tissue, the less chance there is for inflammation and calcification. In addition, if GA is not uniformly distributed throughout the collagen, the sites of the matrix with fewer crosslinks will be subject to faster degradation. Therefore, it is important not only to minimize the amount of GA introduced in the implant, but also to attain a homogeneous distribution of crosslinks throughout the thickness of the collagen.

From experiment A it can be concluded that applying heat during GA fixation of collagen is indeed an effective method of enhancing crosslinking. After 5 min of fixation at 0.01%, or 1 min at 0.1% GA, T_s increased to around 70 °C and 72 °C, respectively, correlating with 77% and 79% of the maximum attainable shrinkage temperature ($\approx$ 90 °C) employing glutaraldehyde tanning. After 5 min of fixation at 0.1%, T_s increased to 85.1 °C (93% of the maximum possible T_s).

In experiment B it was found that concentration, temperature and fixation time were almost equally effective in increasing the level of T_s throughout the full thickness of the collagen sandwich.

These observations accord well with empirically established rules for GA fixation of collagen [19]. If one parameter was increased, increase of a second or third had little or no additional effect as measured by T_s. Apparently, a T_s near maximum for synthetically formed collagen was readily attained using mild conditions.

However, the increasing T_s towards the central sheets of the multilayer model cannot be explained by these rules.

For reasons of reproducibility, we used only free monomeric GA in the starting solution, with no detectable GA polymer. However, during the crosslinking reaction, polymerization has been found to be needed to bridge larger gaps between two reaction sites [5], since the GA molecule cannot even bridge the intramolecular gaps (up to 1.5 nm) in the collagen molecule. Polymerization of GA is known to be enhanced by high concentration (> 0.5%), alkaline pH, and prolonged high temperature [7]. In addition, the polymerization of GA is catalysed by the amino groups of the collagen. Consistently, the conditions in our experiments under which polymer formation is expected to be most enhanced, yielded generally the highest average shrinkage temperatures. The additional T_s increase towards the centre sheets in the diffusion model may then be explained by even more favourable conditions for polymerization in the central part of the model. This also agrees with the finding that extending the fixation period produced more curvature in the centre of the profiles.

In experiment C the same phenomenon, but less pronounced, was observed in native collagen membrane which consists of very densely packed collagen fibres. Over a distance of 125 μm, T_s increased 2 °C, whereas in the synthetically formed collagen sheets, T_s increased 4 °C over three sheets of 40 μm thickness under the same crosslinking conditions (1.0%, 24 h, 45 °C), thus proving the validity of our multilayer model.

Cheung and Nimni [5] found that with relatively high concentrations (0.5% or more) the nature of the crosslinked network so formed was different with fixation at lower concentrations. Above 0.5% GA a fast polymerization reaction takes place at the surface of the collagen matrix, while below 0.5% GA a slow time-dependent reaction occurs, which eventually results in a slightly higher shrinkage temperature [6]. This provided the suggestion that polymerization is necessary for crosslink formation over greater distances. If this suggestion is correct, the disadvantages of polymeric GA, in terms of instability of the polymers and the putative role of the monomers for the induction of inflammatory reactions and accelerated matrix degradation, should be seen in the light of polymer formation being necessary for more complete crosslinking and higher T_s. It would therefore seem that (if these two conflicting roles for polymeric GA do operate) both factors have to be compromised and that T_s as a measure for crosslink density is not a useful indicator *per se* of implant performance in the case of GA fixation.

From experiment D (Fig. 5) it can be concluded that in 5 min, while GA has penetrated the outer collagen sheets producing a substantial rise in shrinkage temperature (18–20 °C), the central sheets are only partly crosslinked, as shown by moderate increase in T_s (10–12 °C). In this experiment, both methods of heating were almost equally ineffective, with only a minor advantage in the faster heating rates achieved by microwaves in such samples. In the short term, the tanning is apparently controlled by diffusion of GA. When the model was first soaked at low temperature (at room temperature or 0 °C) to allow for GA diffusion, probably prior to crosslinking and subsequently heated for 1.5 or 3 min, the resulting flat profiles (Fig. 6) indicated a homogeneous distribution of crosslinks throughout the sandwich.

We conclude that high temperature promotes crosslinking of collagen by GA and allow the use of lower GA concentrations. If GA has not yet diffused throughout the full thickness of the collagen bulk before most of the crosslinking reaction takes place, this will result in an uneven distribution of crosslinks throughout the collageneous material. To avoid such heterogeneous tanning, presoaking the material at low temperature with subsequent short-time high-temperature heating appears to be a good alternative. The heating step is best performed in a temperature-controlled microwave oven, which produces rapid, homogeneous heating and hence short exposure times.

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